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Isolation of 6-Pyruvoyl-tetrahydropterin Synthase cDNAs from Human Brain

Claudia Kluge, Walter Leimbacher, Claus W. Heizmann, Nenad Blau and Beat Thöny[§]

Division of Clinical Chemistry, Department of Pediatrics, University of Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland

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Introduction

The recent update summary of tetrahydrobiopterin deficiency due to autosomal recessive mutations in 6-pyruvoyl-tetrahydropterin synthase (PTPS) compiled 186 cases in total, i.e. 156 cases with the more common *severe* form and 30 cases with the rare *mild* or *peripheral* form (1). Patients with the mild type of deficiency, as opposed to the severe form, do not show any abnormalities of monoamine neurotransmitter metabolites in CSF and thus do not require replacement therapy with L-DOPA and 5-OH-typtophan. Genetic as well as biochemical properties of the normal human PTPS in comparison with mutant alleles might be a key to the understanding of these phenotypical differences. As an example, tissue-specific splicing was uncovered for the Drosophila *purple* gene that expresses the fly PTPS activity (2). The single *purple* gene was shown to express two transcripts with the same putative coding region from two different promoters; i.e. a weak constitutive body promoter and a strong transient head-specific promoter, expressing the alternatively spliced mRNAs with exon structures a-b1-c-d-e and b2-c-d-e, respectively. Interestingly, the *molecular* characterization of the first patient with the mild form of PTPS deficiency revealed one allele, R16C, with the corresponding mutation located in exon 1 in the single human gene (3,4). In the case of a similar tissue-specific splicing as observed for the Drosophila *purple* gene, a brain-specific, non-mutated 5' exon might be present in the human neuronal PTPS-cDNA.

In this work we addressed the question whether the brain-specific form (s) of the human PTPS-cDNA is identical to the liver-specific form. We first isolated PTPS-cDNAs from human brain libraries by using the liver-specific cDNA as a probe. As we did not find any clones with 5'-ends comparable to the known liver cDNA, we applied RACE technology to examine 5'-stretches from freshly prepared RNA of neuronal cells expressing the active PTPS enzyme. Analysis of these amplified cDNAs revealed that the neuron-specific PTPS was identical to the liver-specific form. These findings argue against e.g. tissue-specific splicing as a molecular mechanism to develp the rare peripheral form of PTPS deficiency as opposed to the more common central form.

Materials and Methods

Screening of λgt11-vector derived human brain cDNA libraries (hypothalamus 5'-stretch, Clontech HL1172b and Alzheimer patient, Clontech HL1028) was carried out following conventional protocols. As a probe, the human liver PTPS-cDNA was hybridized at a temperature of 62°C.

To isolate 5' ends of the PTPS cDNA, a standard protocol describing the 'rapid amplification of cDNA ends' (5'-RACE) by Frohman et al. was applied (5). The two PTPS-specific primers PTPS7B

§ Author to whom correspondence should be addressed.

(5'-GGCACATCCATATCCAG-3') and PTPS9 (5'-CGGGATCCGGGGCTGCATAA-TCGCCTCC-3') were used for the first and second round, respectively, of PCR amplification. As a template, we used reverse transcribed total RNA (2 µg) prepared from the cultured human neuroblastoma cells SK-N-BE. Restriction fragments from positive λ-phage clones or from 5'-RACE products were cloned into pUC18/19 plasmids and sequenced using the AutoRead sequencing kit and an Automated Laser Fluorescent (A.L.F.) DNA sequencer from Pharmacia Biotech.

Results and Discussion

Previous Western blot analysis of PTPS-cross-reactive material in extracts from human brain tissues (hypothalamus, mesencephalon, hypophysis) and the neuroblastoma cell line SK-N-BE suggested the presence of the same PTPS in neuronal tissue as in human liver cells or fibroblasts. Similarly, Northern blot analysis of human SK-N-BE (neuroblastoma cells), Hep G2 (hepatoma cells) and fibroblasts showed no difference in PTPS transcript size between the different RNA extracts (unpublished observations). Nevertheless, we performed extensive screening of two commercially available human brain cDNA libraries, Alzheimer patient and hypothalamus, with the liver PTPS-cDNA as a probe and isolated three clones with different 5'-and 3'-ends (Fig. 1). Apart from the fact that none of these isolated had a poly-A tail, each exhibited a different length of its 3'-end. While the longest clone had an extension of 415 bp downstream from the TAG stop codon, the two other clones matched either the previous cDNA isolates from liver (6) or leukemia cells (7) with an extension of 198 bp and 249 bp, respectively, of untranslated DNA. Comparison of the 3' untranslated sequence of the *PTS* gene revealed that the entire 415 bp are part of exon 6. Moreover, polyadenylation signals, a classical (AATAAA) and a non-classical (ATTAAA), were present 15 bp and 16 bp upstream of the 3' end of the clones with the 415 bp and 249 bp non-coding DNA, respectively. These findings suggest that up to 3 different 3'-ends of the PTPS transcript may be present in neuronal and other human tissues.

Regarding the 5'-ends of the cDNA library isolates, the two longer clones were deleted for exon 3, and only one extended into exon 1. A deletion of the 23 bp in the PTPS-cDNA, comprising exon 3 on genomic DNA, leads to a frame shift

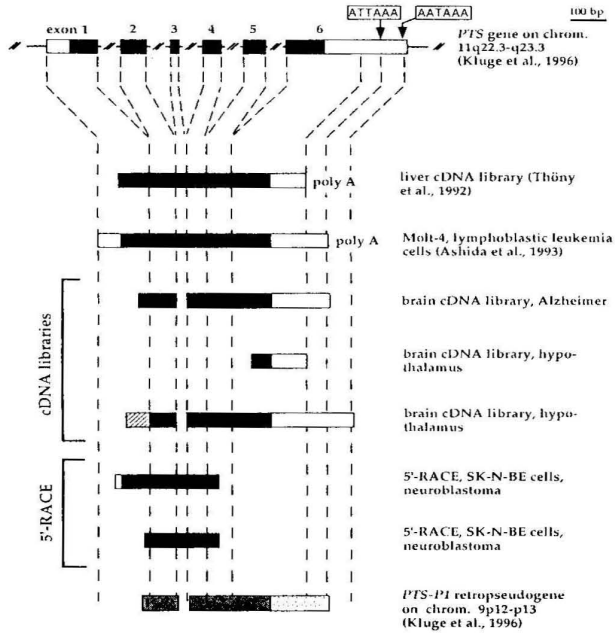

Figure 1. Organization of the human *PTS* gene and extension of corresponding cDNA clones isolated from various sources. The *PTS* gene located on chromosome 11q 22.3-q23.3 is composed of 6 exons as indicated by the boxes. The coding regions are in black, whereas the 5'-and 3'-untranslated stretches are depicted as open bars. The position of the two putative polyadenylation signals (ATTAAA and AATAAA) are indicated by arrows. The different sources of individual cDNA isolates are given on the right. Polyadenin stretches found at the 3'-ends of cDNAs are indicated by 'polyA'. The hatched box is a spliced fragment which originates from intron 1 (see text for details). For comparison, the extension of the 74%-identiy to the *PTS-P1* retropseudogene is given at the bottom.

and a subsequent stop codon. A similar mutant cDNA allele has been isolated previously from a PTPS patient (K54X allele; 8). The cause for expressing such a mutant RNA was a genomic T to A transversion at position-7 in the 3'-acceptor splice site of the patient's intron 2, which leads to exon 3 skipping (4). Interestingly, a sequence alignment between the liver PTPS cDNA and the *PTS-P1* retropseudogene revealed a significant homology gap of 23 bp extending over the exon 3 region. This implicates that exon 3 skipping does not occur exclusively due to 3'-acceptor splice mutations, but might also be caused by a splicing polymorphism.

In addition to a deletion of exon 3, we found in one clone from the hypothalamus cDNA library a DNA fragment which was fused at the 5'-end of exon 2. This fragment turned out to be an internal 226 bp stretch from intron 1 (nucleotide

position 1451-1676 in the deposited GenBank sequence L76259). This cDNA clone, which is a splice product consisting of the 226 bp 'exonic' fragment plus exons 2, 4, 5 and 6, did also not yield any open reading frame in its 5'-portion of the cDNA.

Although at this stage we could not exclude any functional role for the isolated brain cDNA clones, we assumed that the exon 3 deletion in two clones and the spliced fragment from intron 1 in one clone were due to some artifacts present in the neuronal cDNA libraries analyzed. Furthermore, as we detected PTPS enzyme activity (9) and cross-reactive material in human hypothalamus extract (not shown), we expected to find a cDNA that encodes a functional PTPS. To discriminate between erroneously spliced cDNAs present in the brain cDNA libraries and potentially alternative splicing in neuronal cells, we carried out 5'-RACE experiments with freshly prepared RNA from cultured neuroblastoma cells that expressed the human PTPS endogenously (SK-N-BE cells, unpublished observation). The PTPS-specific downstream primers were selected to amplify the cDNA upstream from exon 5 (PTPS7B and PTPS9; for details see Materials and Methods). The 5'-RACE products were gel purified, subcloned into plasmid vectors, and sequenced. The two clones that we analyzed did neither show any alternatively spliced forms of PTPS-cDNA, nor a deletion of exon 3 (Fig. 1). Furthermore, one clone had the complete coding sequence of exon 1 plus 18 bp of the non-coding 5'-upstream sequence. Unfortunately, we did not isolate any clones that were extended up to the previously described 68 bp upstream from the translational start site (Ashida et al., 1993).

In summary, our findings argue against alternative 5'-exons in the human *PTS* gene for expression of a functional enzyme, since no such tissue- or cell-specific transcripts for the human PTPS were isolated. Whether the occurrence of a deletion of exon 3 in processed transcipts is a polymorphism or has some functional meaning needs to be clarified. Furthermore, to unravel the molecular basis of clinical and biochemical differences of the various forms of PTPS insufficiency, more data about the molecular defects in patients especially with the peripheral type of PTPS deficiency are required.

Acknowledgments

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